Gel Electrophoresis

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Introduction

The separation of polypeptides by electrophoresis allowed Arne Tiselius to describe protein fractions corresponding to albumin, α-, β-, and γ-globulins in serum with the first published diagram of human serum protein electrophoresis in 1939. The number of fractions slowly expanded into electrophoretic subfractions identified as α_1, α_2, β_1, β_2, γ_1 and γ_2. The mobility characteristics of these fractions are still used to denote serum proteins such as α_1-macroglobulin, α_2-antiplasmin or β_2-microglobulin. Sophisticated new electrophoretic techniques for identifying many proteins simultaneously and relating them to diseases have been developed over the years, many of them being described in other studies. Almost all body fluids have been studied by electrophoresis, serum, urine and cerebrospinal fluid being evaluated in great detail by different techniques. However, despite the major developments and progress achieved in protein separation, only a restricted number of methods are routinely used in the clinical laboratory. Nowadays, serum protein electrophoresis is mainly used to study major serum protein alterations such as those observed in patients with inflammatory, liver or kidney diseases, as well as in patients presenting lymphoproliferative disorders and alterations of immunoglobulin (Ig) production. Electrophoretic techniques, in association with ultracentrifugation, are also used to study lipoproteins and to classify hyperlipidaemic disorders.

Here, we provide selected clinical situations studied by electrophoretic methods, with a particular emphasis on 2D-PAGE, in order to illustrate how electrophoresis can be used to gain insight into particular clinical problems.

Monoclonal Immunoglobulins

The large diversity of antibodies in an individual results from highly complex mechanisms influencing B-cell development, expansion, and immunoglobulin (Ig) secretion. This diversity is so complex that electrophoretic techniques applied to sera from healthy adults cannot separate individual clonal products. Immunoglobulin produced by an expanding B cell clone to a level permitting detection by electrophoretic techniques is known as 'monoclonal gammopathy' (MG), and has been observed in a wide variety of disease. MG can be also observed in humans without overt disease and is termed 'benign MG', or 'monoclonal gammopathy of undetermined significance'. The frequency of benign MG has been increasing in parallel with the refinement of the techniques. Identification of monoclonal immunoglobulins requires sensitive and rapid screening methods. Electrophoresis on cellulose acetate membrane is satisfactory for initial screening.

High-resolution agarose gel electrophoresis (HRE) is more sensitive for the detection of rare monoclonal proteins (**Figure 1**). Confirmation of the presence of an MG should be performed using methods such as immunoelectrophoresis (IEP) or immunofixation electrophoresis (IFE). IEP was described in 1953, and, until recently, was considered as the method of reference. As a routine procedure, it is time-consuming, and requires a high level of technical expertise. The consequence of the long diffusion process is that small amounts of protein become too dilute to be detected, resulting in low sensitivity. The most important drawback of IEP is the so-called 'umbrella effect'. Furthermore, the demonstration of κ and λ monoclonal light chains belonging to IgA or IgM isotypes is often masked by the presence of polyclonal IgG, that, because of faster diffusion, migrates ahead to an area situated between IgA and IgM. IFE, like IEP, includes two steps, but without the diffusion process. In the first step, protein fractions are separated by agarose gel electrophoresis; in the second step, they are revealed by overlaying of antiserum specific to the different immunoglobulin heavy and light chains. The technique is considered as the procedure of choice in the routine diagnosis of MG, because of its simplicity, speed, and sensitivity. Other advantages of the method are ease of interpretation and the absence of diffusion. Furthermore, the enhanced sensitivity of IFE also yields more frequent detection of low concentration MG and, what is more important, of multiple immunoglobulin bands or subtle bands of restricted heterogeneity. In such cases, further assessment of the clonality of immunoglobulins depends on additional techniques such as immunoblotting, immuno-isoelectric focusing or high-resolution two-dimensional polyacrylamide gel

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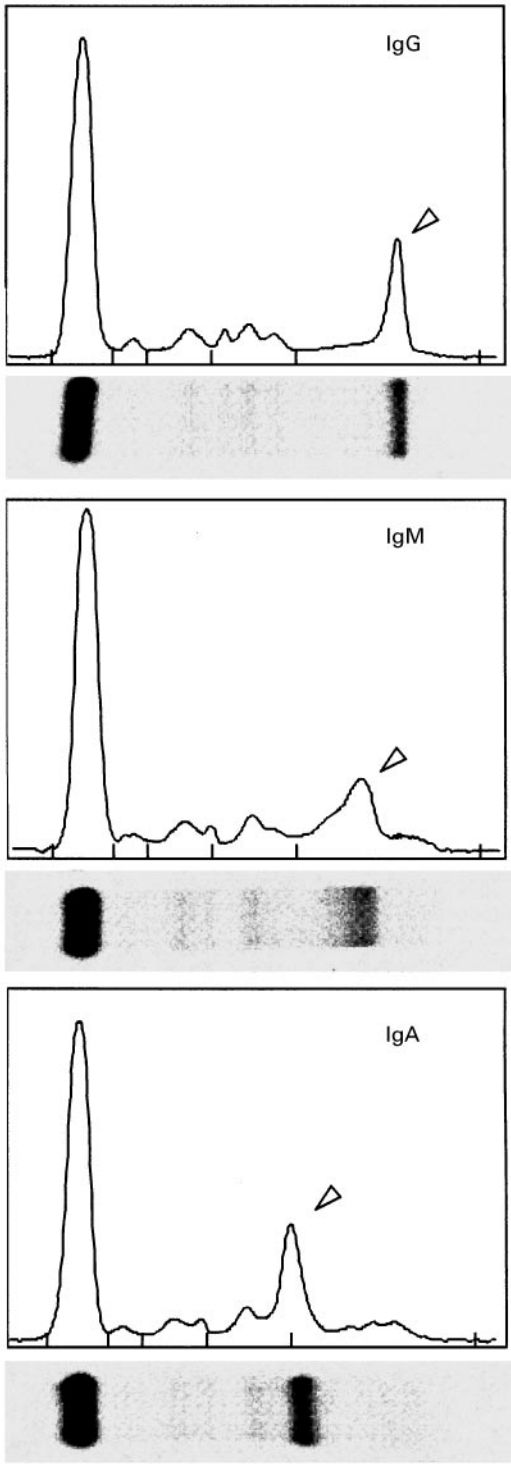

Figure 1 High resolution serum protein agarose electrophoresis profiles. Serum protein electrophoresis, and protein densitometry of serum samples from patients presenting monoclonal IgG-λ, IgM-λ and IgA-λ gammopathies were performed by using commercially available kits. The isotype of the monoclonal heavy chains as well as the type of the monoclonal light chains were identified using immunofixation electrophoresis. The protein peaks corresponding to the monoclonal components are shown by arrowheads.

electrophoresis (2D-PAGE). With 2D-PAGE, α, μ, γ and δ chains appear in nonoverlapping regions of protein maps (**Figure 2**). Monoclonal heavy chains are easily differentiated from polyclonal heavy chains, according to their different two-dimensional electrophoretic patterns. Polyclonal heavy chains, according to their different two-dimensional electrophoretic patterns. Polyclonal heavy chains are highly heterogeneous and are resolved as 'unspotted' diffuse zones whereas monoclonal heavy chains show charge and, to a lesser degree, size microheterogeneity.

Polyclonal light chains appear as clustered nondiscrete spots following 2D-PAGE, and form cloudy zones with unevenly distributed densities. In about two-thirds of patients with MG, monoclonal light chains are detected as a dominant and well-defined spot. In the remaining one-third of the patients, examined monoclonal light chains disseminate in more than one spot. In patients with monoclonal heavy chain disease, only fragments of monoclonal heavy chains are synthesized and released into the circulation by malignant lymphoplasmocytic cells. Such fragments are identified with immunofixation electrophoresis as proteins bands that only bind specific anti-heavy chain antibodies, but not specific anti-κ or anti-λ light chain antibodies (**Figure 3**).

However, the abnormal characteristics of such monoclonal heavy chains are best studied using 2D-PAGE of protein-G purified fractions, as demonstrated in **Figure 4**. A set of spots (γ') is observed at the basic side of the gel, in an area corresponding to p*I* from 5.8 to 6.8, and M_r of 32 to 38 kDa. Spots corresponding to polyclonal IgG γ chains and polyclonal immunoglobulin light κ and λ chains are also identified. No spot corresponding to a monoclonal immunoglobulin light chain is observed. The 32 to 38 kDa M_r of the monoclonal γ' chain indicates that it is a fragment of a normal γ chain, and the microheterogeneities of the p*I* suggest that it is glycosylated. Finally, the binding of the molecule to protein G implies that the Fc region of the immunoglobulin is intact. In contrast to what was seen in the serum of another patient presenting the same disease, abnormal monoclonal γ chains of higher M_r are not observed.

Cold Agglutinins and Cryoglobulins

Cold agglutinins (CAs) are immunoglobulins, most frequently IgM, that agglutinate erythrocytes at temperatures below 37°C. Immunohaemolytic anaemia related to CAs can be observed in a wide variety of diseases (**Figure 5**).

Monoclonal CAs, generally belonging to the IgM isotype, are observed in patients with lymphopro-

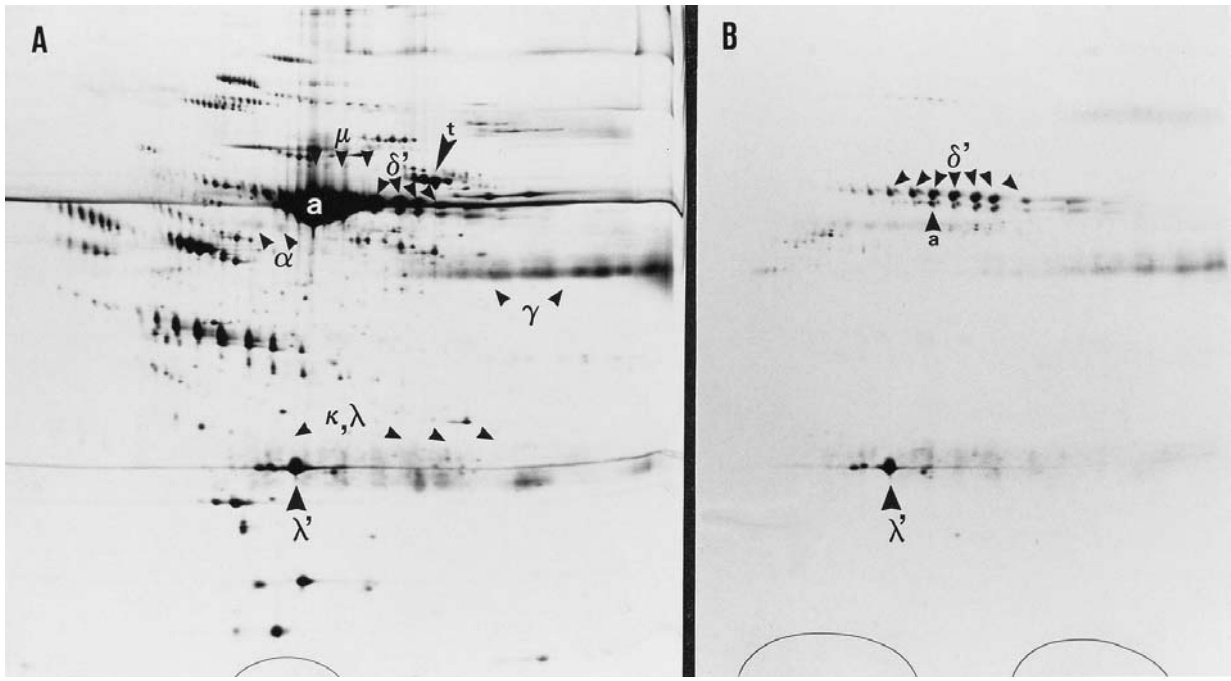

Figure 2 High-resolution two-dimensional polyacrylamide gel electrophoresis in a case of monoclonal IgD-λ. (A) Serum and (B) purified IgD (anti-λ Sepharose). a, albumin; μ, polyclonal heavy chains of IgM; α, polyclonal heavy chains of IgA; γ, polyclonal heavy chains of IgG; κ,λ, polyclonal immunoglobulin light chains; δ′, monoclonal δ-chain monoclonal; λ′, monoclonal λ chain; t, transferrin. The gels were silver stained, and presented with the higher molecular weights at the top, and the acidic side on the left. (Reproduced from Tissot JD, Schneider P, Hohlfeld P *et al.* (1993) Two-dimensional electrophoresis as an aid in the analysis of the clonality of immunoglobulins. *Electrophoresis* 14: 1366, with permission from Wiley-VCH, Weinheim.)

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liferative disorders or suffering from the 'idiopathic' chronic cold agglutinin disease, whereas polyclonal CAs, also belonging to the IgM isotype, may be found in patients with various infectious diseases, but more particularly in patients with Epstein–Barr virus or *Mycoplasma pneumoniae* infection. The evaluation of the clonality of CAs, that are frequently at very low concentrations in serum, can be easily performed using 2D-PAGE. CAs are isolated from serum by cold absorption of immunoglobulins on red bloods cells. After several cold washes, red blood cells coated with CAs are rewarmed to 37°C. After centrifugation, the supernatant is collected and studied with 2D-PAGE. As mentioned in the previous section, polyclonal IgM are quite easily differentiated from monoclonal IgM according to their different electrophoretic patterns (**Figure 6**).

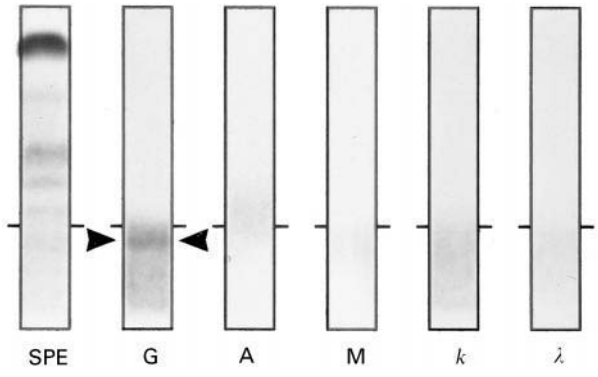

Figure 3 Immunofixation electrophoresis in a case of γ-chain disease. A serum sample from a 56-year-old patient was analysed by immunofixation electrophoresis. A protein band (arrowheads) was shown to react with anti-human immunoglobulin chain antiserum, but not with anti-human κ or λ light-chain antibodies. SPE, serum, protein electrophoresis; G, A, M, κ, λ, immunodetection of protein bands with corresponding antisera.

Cryoproteins are defined as proteins precipitating at low temperature. Most frequently, the precipitate contains immunoglobulins, and are therefore called cryoglobulins. Three types of cryoglobulins have been described: type I contains a single monoclonal immunoglobulin, whereas type II is a mixture of a monoclonal immunoglobulin with polyclonal immunoglobulins, and type III is a mixture of polyclonal immunoglobulins of different isotypes, most frequently IgG and IgM (**Figure 7**). Type II and type III are also called mixed cryoglobulins. A new type II–III class of cryoglobulins, containing polyclonal IgG associated with a mixture of polyclonal and monoclonal IgM has been recently described through 2D-PAGE. In a series of 265 cryoglobulins studied with 2D-PAGE, we identified type I, II, II–III, and III in 3.4%, 26, 22.6 and 43.8% of the cases, respectively.

Figure 4 High resolution two-dimensional polyacrylamide gel electrophoresis in a case of γ-chain disease. Protein G purified protein fraction from a 56-year-old patients presenting γ-chain disease was analysed using 2D-PAGE. γ, polyclonal heavy chains of IgG; γ', spots corresponding to monoclonal γ chain fragments; κ-λ, polyclonal immunoglobulin light chains. The gel was silver stained, and presented with the higher molecular weights at the top, and the acidic side on the left.

They were composed of oligoclonal immunoglobulins mixed with traces of polyclonal IgG in 4.2% of the cryoglobulins studied. These cryoproteins were tentatively named type II–III$_{variant}$ cryoglobulins.

Fetal Proteins

Gene expression and regulation are dependent on highly sophisticated mechanisms. The genome-related modifications of protein synthesis and expression are most evident during fetal development.

The development of a safe and easy technique for fetal blood sampling has greatly improved our knowledge of normal fetal physiology, including some aspects of protein biology. However, the amount of pure fetal blood which can be derived for research purposes from samples obtained for prenatal diagnosis is obviously limited. Thus, electrophoretic techniques that allow studies of plasma and serum proteins contained in minute samples (usually less than 1 μL) must be applied. Many proteins detected in the plasma and serum of adults are already present in the blood of normal embryos at a very early stage of gestation. Not all proteins are produced by the conceptus but many are the result of transfer from the mother across the placenta. Some proteins remain at a fraction of their adult levels throughout intrauterine life, others are progressively produced in increasing amounts when term approaches, and finally, others are present in higher concentrations during fetal life than after delivery. Modifications of plasma and serum protein concentrations during early extrauterine life have been well documented. The plasma concentration of a given protein is governed not only by fetal synthesis and degradation, but also by the result of the exchange between mother and fetus through the placenta. Materno-fetal transfer of proteins involves several different mechanisms such as a first-order process or active transport. The concentration of each fetal plasma protein results of a balance between opposing dynamic metabolic and physiological processes which proceed simultaneously. The relative impact of these factors contributing to a plasma protein concentration continuously shifts during development, and not always in the same manner. 2D-PAGE is an ideal tool to study such a dynamic process. Fetal protein maps reveal spots which are always absent in those of adults. A readily evident set of spots, located at the acid side of albumin is found in such fetal samples and corresponds to α-fetoprotein (**Figure 8**). This protein is less apparent as gestational age increases. A second 'fetal' polypeptide, characterized by an apparent M_r of 46 kD and a p*I* of 5.0 is observed as a small spot, located under those of α_1-antitrypsin. *N*-terminal microsequencing (25-EDPQ) and immunoblotting using specific anti-α_1-antitryspin antibodies reveal that this spot most likely

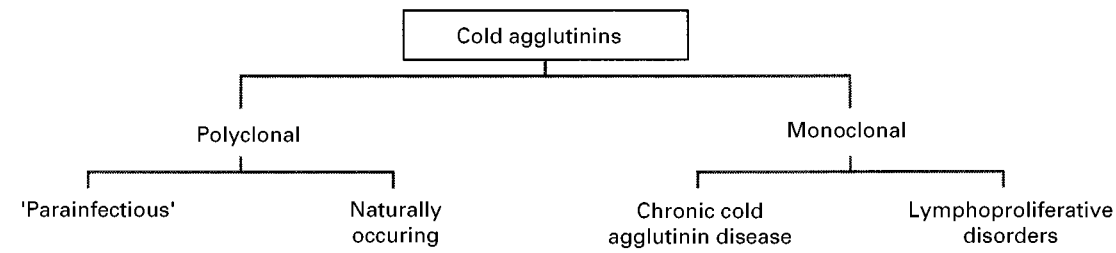

Figure 5 Classification of cold agglutinins.

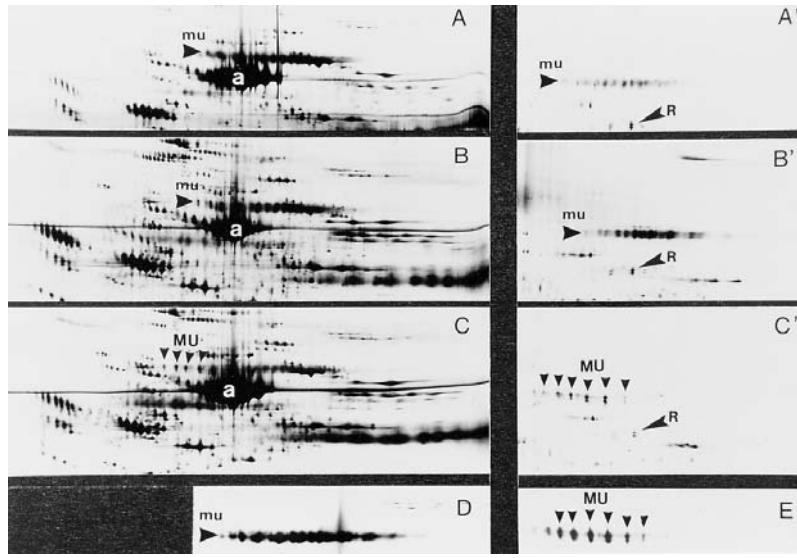

Figure 6 Characterization of cold agglutinins using high resolution two-dimensional polyacrylamide gel electrophoresis. Details of electrophoretograms of plasma/serum samples (A–C) and their purified cold agglutinins fractions (A′–C′). Purified polyclonal (D) and monoclonal (E) IgM heavy chains are shown as controls. A–A′, serum and purified CAs from a patient with chronic virus C hepatitis; B–B′, plasma and purified CAs from a patient with *Mycoplasma pneumoniae* infection; C–C′, plasma and purified CA from a patient with chronic cold agglutinin disease. a, albumin; R, reference protein used to highlight the relatively acidic p*I* of the monoclonal μ-chain found in patient C; mu, polyclonal μ chain; MU, monoclonal μ chains. The charge microheterogeneity of monoclonal μ chains is highlighted by arrowheads. The gels were silver stained, and presented with the higher molecular weights at the top, and the acidic side on the left. (Reproduced from Tissot JD and Spertini F (1995) Analysis of immunoglobulin by two-dimensional gel electrophoresis. *Journal of Chromatography A* 698: 225–250, with permission from Elsevier Science.)

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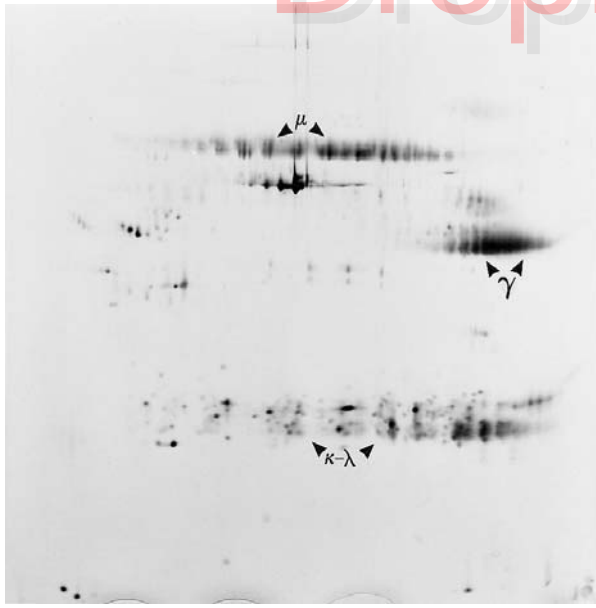

Figure 7 High-resolution two-dimensional polyacrylamide gel electrophoresis of mixed type III cryoglobulins. The cryoprecipitate is characterized by the presence of a mixture of polyclonal IgM and polyclonal IgG. μ, polyclonal IgM μ chains; γ, polyclonal IgG γ chains; κ-λ, immunoglobulin light chains. The gel was silver stained, and presented with the higher molecular weights at the top, and the acidic side on the left.

corresponds to a fetal-specific form of α_1-antitryspin, already identified in mouse plasma. This polypeptide is observed in all fetal samples and in all samples obtained from infants of under two years of age but is either undetectable or appears as a shaded spot in adults.

Genetic polymorphism of some serum proteins such as Gc-globulin, Apo A-IV, or Apo E (**Figure 9**) can be also studied using 2D-PAGE. As shown in **Table 1**, the technique allows determination of the frequency of the Apo-E phenotypes, as well as the calculation of the gene frequencies.

During fetal development, 2D-PAGE reveals a progressively more and more complex protein pattern and an increase in the size of many spots. Three major protein pattern modifications are observed on 2D gels of fetuses at different gestational ages: (a) the progressive appearance of the protease inhibitor α_1-antichymotrypsin; (b) the progressive increase of polyclonal IgG, which is particularly evident during the last weeks of pregnancy; (c) the gradual diminution of α-fetoprotein which is undetectable on protein maps of term newborns. On the other hand, before the 38th week of gestation, polyclonal IgA or IgM heavy chains, as well as the two main proteins involved in free haemoglobin transport and catabolism — haptoglobin and hemopexin — are not detectable.

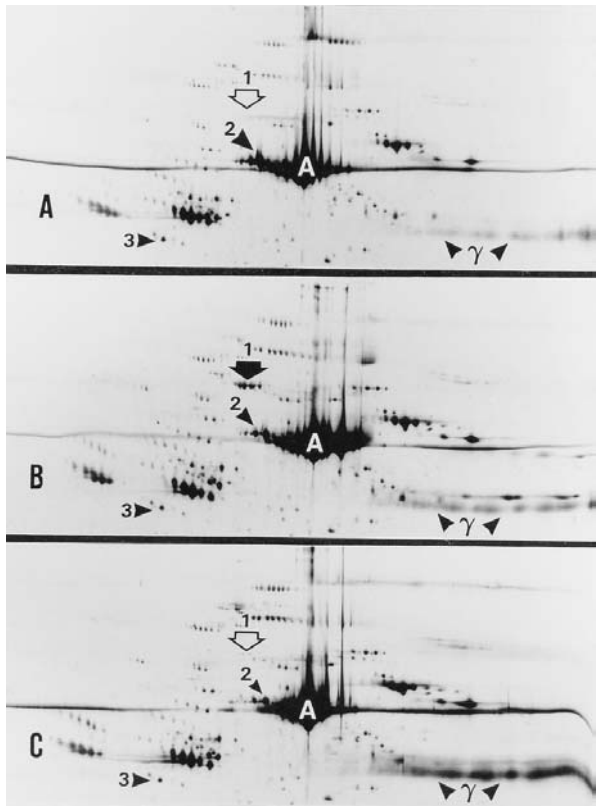

Figure 8 Fetal proteins. Details of electrophoretograms of plasma samples obtained from fetuses at 22 (A), 28 (B), and 32 weeks (C) of gestation. A, albumin; γ, immunoglobulin gamma chains; 1, dimers of fibrin (γ chains), only observed when small clots were present in the sample; 2, α-fetoprotein; 3, fetal form of α_1-antitrypsin. The gel was silver stained, and presented with the higher molecular weights at the top, and the acidic side on the left.

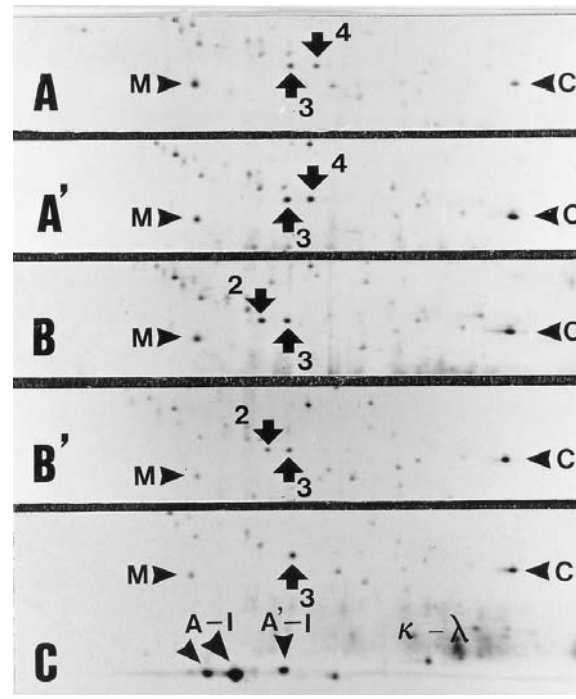

Figure 9 Genetic polymorphism of apolipoprotein E in fetuses. Phenotype E3/4 (A, fetus at the age of 22 weeks, A′ fetus at the age of 30 weeks), phenotype E2/3 (B, fetus at the age of 22 weeks, B′ fetus at the age of 30 weeks), phenotype E3/3 (C, fetus at the age of 22 weeks). M, α_1-microglobulin; C, γ chain of complement factor C4; A-I, apolipoprotein A-I, A′-I, pre-apolipoprotein A-I; κ and λ, immunoglobulin light chains. The gel were silver stained, and presented with the higher weights at the top, and the acidic side on the left.

Carbohydrate-deficient Glycoprotein Syndrome (CGDS)

Carbohydrate-deficient glycoprotein syndrome (CDGS) is a group of autosomal recessive disorders affecting multiple organ systems. All of the affected patients present moderate or severe brain disorders. In the first years of life, CDGS is characterized by hypotonia with failure to thrive, dysmorphism and coagulation abnormalities. These clinical manifestations are the direct embryologic and physiological consequences of an underglycosylation of protein. The alterations are related to a reduced number of entire *N*-glycans on glycoproteins and they have been described for serum transferrin, α_1-antitrypsin, α_1-acid glycoprotein, α_1-antichymotrypsin, fetuin, ceruloplasmin and antithrombin III, C3a and C4a. In patients with CDGS type 1a and type 1b, the post-translational defect is due either to a deficiency of phosphomannomutase or a deficiency of phosphomannose isomerase. Both these defects alter the metabolism of mannose in cells and result in a decrease of GDP-mannose for *N*-glycan synthesis. The diagnosis is usually made by isoelectric focusing of serum transferrin showing a different pattern of cathodal shift due to the loss of terminal sialic acids. Another way of diagnosis is to separate the transferrin isoforms lacking their *N*-glycans by using SDS-PAGE. Other more complex diagnostic methods have been

Table 1 Distribution of ApoE in fetuses and adults determined by 2D-PAGE

Phenotypes	*Fetuses*	*Adults*	*Total*[a]	*Expected*[b]
E2/2	0	2	2	1
E2/3	10	21	31	31
E2/4	1	1	2	4
E3/3	76	152	228	225
E3/4	18	32	50	57
E4/4	2	6	8	4
Total	107	214	321	322

[a]Allele frequencies: Apo E*2: 0.058, Apo E*3: 0.836, Apo E*4: 0.106.
[b]Hardy-Weinberg equilibrium.

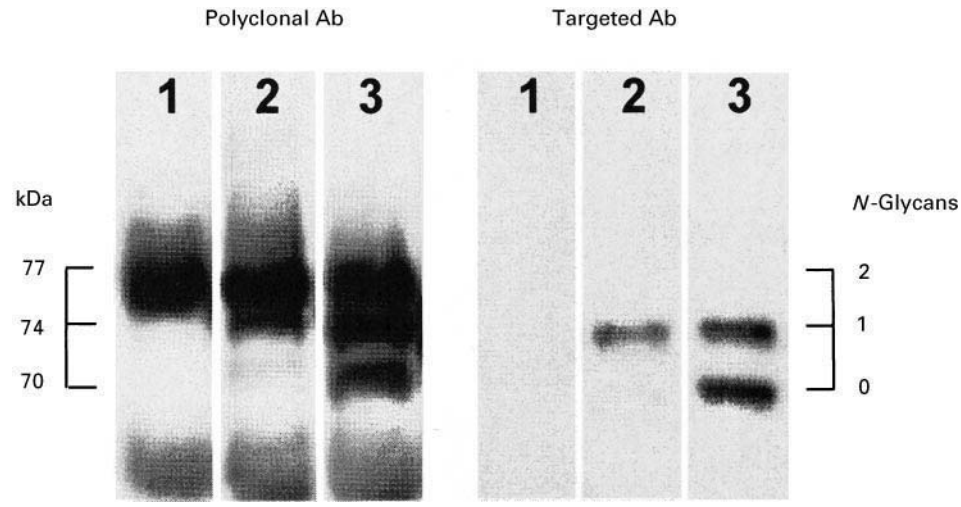

Figure 10 Comparison of the specificity of antibodies raised against transferrin. Transferrin isoforms identified by antibodies directed against multiple epitopes (polyclonal Ab) and transferrin isoforms that presented the *N*-432 transferrin epitope recognized by a targeted antibody (targeted Ab). Lane 1, normal serum control; lane 2, serum from a patient with alcoholism; lane 3, serum from a patient with type 1 carbohydrate-deficient glycoprotein syndrome (CDGS).

reported such as 2D-PAGE and matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry of serum transferrin.

We have described the characterization of a new antibody which is specifically directed against the *N*-glycan binding site localized on the asparagine-432 of transferrin and which allows immunodetection of transferrin devoid of *N*-glycan (**Figure 10**). This antibody has the potential to provide a new immunochemical tool for the CDGS diagnostic.

Conclusion

Numerous diseases can be diagnosed using electrophoretic analyses of body fluid proteins. However, many different electrophoretic techniques are available. Electrophoresis on cellulose acetate is employed to study many different enzymes such as serum amylase, pancreatic isolipase, and pyruvate kinase. Agarose gel electrophoresis is extensively used to gain insight into the understanding of lipoprotein biology. Isotachophoresis or 'displacement' electrophoresis allows the simultaneous concentration and effective separation of different charged substances, including biological macromolecules. Affinity electrophoresis is used to evaluate cross-reactivity of antihapten antibodies whereas lectin affinity electrophoresis is best used to characterize serum glycoproteins. Capillary electrophoresis offers the possibility of rapid and automated analysis of different kinds of molecules, with high reproducibility and improved quantification. Isoelectric focusing as well as SDS-PAGE are cornerstone techniques used to characterize polypeptides. 2D-PAGE is increasingly used as an important tool for biological research. However, despite the fact that these techniques are particularly useful for studying a clinical problem, routine clinical analysis is frequently incompatible with the use of such sophisticated techniques. As illustrated by several examples presented in this article, 2D-PAGE can be chosen as a complementary technique to gain insight into several specific clinical problems. The combination of different electrophoretic techniques can be very useful to resolve complex problems.

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Further Reading

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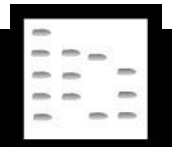
CLINICAL CHEMISTRY: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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Introduction

Clinical medicine is mainly based on the results of chemical analysis and these results are very important. It is estimated that more than 50% of diagnoses are based on laboratory data. The analyses are usually done using immunoassays, chromatographic and spectroscopic methods. The combined techniques, e.g. liquid chromatography–mass spectrometry (LC/MS) gas chromatography–tandem mass spectrometry (GC-MS-MS) are also applied. The development of sophisticated methods of analyses reduced interest in simple and rapid methods such as thin-layer chromatography (TLC). Starting from paper chromatography and improved over the years, TLC has become very useful also for clinical medicine. TLC has been used to solve analytical, and sometimes preparative, problems.

A large number of publications on the use of TLC for clinical medicine analysis can be found in the literature. An excellent compilation concerning different applications of analytical methods (including TLC) for isolation and identification of drugs in pharmaceutical, body fluids and post-mortem materials is the book edited by Moffat. This work, prepared in the Department of Pharmaceutical Sciences of the Pharmaceutical Society of Great Britain, is a comprehensive and clear account of clinical analytical chemistry up to 1986. An excellent supplement, covering the early 1990s, is the monograph of Jain. More recent works more commonly refer to applications of instrumental TLC.

General Principles of Clinical Medicine Analyses by TLC

TLC is a most economical and cost-effective technique and ideally suited for performing clinical analyses of biological samples, where the sample load is high. The method is characterized by high selectivity, enabling separation of the analyte from interfering substances. At least two basic groups of applications of TLC in clinical medicine analysis can be distinguished. The most important of them are where TLC has been applied for analytical and screening purposes where qualitative or semi-quantitative results are required. The second group concerns work, where TLC performs the function of a clean-up technique for initial sample preparation.

Clinical analysis presents a difficult analytical problem. The proteins, lipids, or carbohydrates, usually present in biological samples, may interfere with analytes. Analyses are also made difficult by the fact that many substances of interest are only slightly soluble in organic solvents.

Sample Preparation

There is a generally accepted view in laboratory practice, that the TLC stage of sample preparation is of little importance because it is merely a clean-up technique. This is incorrect, especially in the case of clinical medicine analysis where analytes are to be found in complex matrices such as plasma, serum or urine, whole blood, faeces, saliva, cerebrospinal fluid, gastric fluid or body tissues. These matrices are very complex multicomponent mixtures and therefore sample preparation prior to analysis cannot be omitted.

The process of separation from biological samples and purification of analytes is usually realized by protein precipitation, dialysis, hydrolysis, ultrafiltration, dilution, liquid–liquid or solid-phase extraction. Originally, the most common extraction method was